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Award Number: DAMD17-98-1-8478

TITLE: Repression of the Androgen Receptor by WT1, a Tumor
Suppressor Gene

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REPORT DATE: February 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE February 2001	3. REPORT TYPE AND DATES COVERED Final (15 Aug 98 - 31 Jan 01)	
4. TITLE AND SUBTITLE Repression of the Androgen Receptor by WT1, a Tumor Suppressor Gene			5. FUNDING NUMBERS DAMD17-98-1-8478	
6. AUTHOR(S) David McConkey, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas M.D. Anderson Cancer Center Houston, Texas 77030 E-Mail: dmcconke@mdanderson.org			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES This report contains colored photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Androgen-independent prostate cancer responds poorly to conventional chemotherapeutic agents. This observation has prompted an aggressive search for the molecular mechanisms responsible for drug resistance in these cells and for new agents that specifically target or overcome these resistance mechanisms. The two Specific Aims of this proposal were directed at both of these important topics. In the first we proposed to identify the molecular mechanisms responsible for overexpression of BCL-2 and associated apoptosis resistance in androgen-independent variants of the human LNCaP prostate adenocarcinoma line selected for enhanced metastatic potential in vivo. The second Specific Aim focused on characterizing the activity and mechanisms of action of a new agent (PS-341) that was found to be capable of bypassing BCL-2-mediated cell death resistance in transfectants in vitro. Our progress toward the first objective revealed that overexpression of BCL-2 dramatically enhanced the activity of the androgen receptor (manuscript submitted). We also demonstrated that PS-341 displays strong activity against established human prostate cancer xenografts. Subsequent analysis of PS-341's interactions with conventional chemotherapy demonstrated that it promotes the activities of DNA damaging agents but interferes with taxoids in a schedule-dependent fashion. Our data have been instrumental in helping NCI/CTEP design upcoming PS-341-based clinical trials.				
14. SUBJECT TERMS Prostate Cancer, androgen-independent, BCL-2, proteasome			15. NUMBER OF PAGES 55	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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A. Introduction

Androgen-independent prostate cancer (AI-PC) remains a serious problem with no effective therapies available at present. The failure to control AI-PC is due in part to the limited number of biological targets attacked by conventional chemotherapeutic agents and to a lack of information concerning the mechanism(s) that mediate therapeutic resistance. In previous work we used derivatives of the human androgen-sensitive LNCaP cell line selected for increased metastatic potential to show that the acquisition of androgen independence is associated with increased expression of the anti-apoptotic protein, BCL-2. We also obtained preliminary evidence that an investigational agent (PS-341) designed to inhibit a novel target (the proteasome) was capable of inducing cell death in AI-PC cells as well as in prostate cancer cells engineered to overexpress BCL-2. These observations served as the bases for the Specific Aims of our proposal, introduced below.

Specific Aim 1: Identify the molecular mechanisms underlying overexpression of BCL-2 and suppression of BAX in androgen-insensitive LN3 cells.

Specific Aim 2: Determine the effects of proteasome inhibitors on tumor growth and apoptosis in vivo.

B. Body

Progress/Results of Studies in Specific Aim 1:

A detailed discussion of the results of the experiments outlined in the original Tasks 1-4 under this Specific Aim was provided in our 18-month progress report. For the sake of brevity, these results are summarized in abbreviated form here. In this Specific Aim we proposed to determine BCL-2 and BAX protein stability (Task 1), analyze rates of transcription (Task 2), conduct promoter-activity measurements (Task 3), and conduct EMSA experiments to characterize the transcription factors involved (Task 4). Pulse-chase studies of BCL-2 and BAX stability proved to be difficult because both proteins exhibit long half-lives in our cells, and the question of whether differential stability contributes to the increased expression of BCL-2 in the LN3 cells remains open for the moment. However, we conducted extensive transcriptional analyses that support the idea that protein stability may be involved. Steady-state mRNA levels (measured by ribonuclease protection) were indistinguishable in our cell panel. In addition, experiments with a luciferase-based, BCL-2 promoter-reporter construct (obtained from Tim McDonnell, M.D. Anderson) revealed no differences in promoter activity in any of the clones. Direct analysis of transcription factor activity by EMSA did reveal one important difference: The LN3 and LN4 clones display substantially increased NFkB activity, a result that was confirmed using a synthetic (3x) NFkB response element-driven reporter construct (see Figure below). This NFkB activation could have important consequences for PS-341 therapy (Aim 2), because proteasome inhibitors are known to be potent inhibitors of the transcription factor. Furthermore, work in Baldwin's laboratory demonstrates that NF_B is also activated in androgen-independent tumors derived from the CWR22 xenograft (M. Mayo, personal communication), strongly suggesting that NFkB activation is a common feature of androgen-independent disease. Experiments designed to more directly test the NFkB-BCL-2 interaction in our cells will be tested in future studies. However, in the course of our experimentation we uncovered a novel interaction between BCL-2 and the androgen receptor, discussed in detail below. Characterizing this interaction and defining the molecular mechanisms involved has become one of our top research priorities. The first manuscript on the subject has been submitted for publication (see Appendix), and a second paper is planned for the near future.

Metastatic LNCaP variants display reduced androgen dependence. Primary tumors are known to be highly heterogeneous at the biological and molecular levels. Dr. Pettaway used an orthotopic selection strategy to isolate subclones of the LNCaP cell line that possessed either increased tumorigenicity at the primary site or increased metastatic potential (1). Two of the subclones (LNCaP-Pro5 and LNCaP-LN3)(1, 2) were selected for further analysis. Cells were grown in conditions of androgen deprivation in charcoal-stripped serum in the presence of the androgen receptor antagonist, bicalutamide (Casodex), and proliferation was measured at 48 h by ^3H -thymidine incorporation. As expected, growth of the parental LNCaP cells was inhibited (Figure 1), consistent with previous work showing that the cells are androgen-responsive. A very similar profile was observed with the LNCaP-Pro5 cells (Figure 1). In contrast, proliferation of the LNCaP-LN3 cells was only slightly affected (Figure 1), indicating that the selection for enhanced lymph node metastasis co-selected for androgen insensitivity. Consistent with this idea, orthotopic tumors derived from the LN3 subclone were also much less sensitive to androgen deprivation in vivo (16). The LN3 cells also secreted higher levels of PSA in vitro and in vivo as measured by ELISA (1).

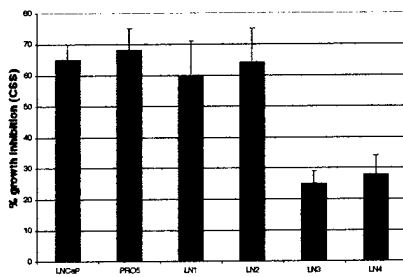


Fig. 1: Growth of LNCaP variants in charcoal-stripped serum. Note that growth inhibition is modest in LN3 and LN4 cells.

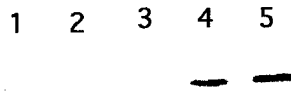


Fig. 2: BCL-2 expression in the LNCaP panel. Lane 1, Pro5; lane 2, LN1; lane 3, LN2; lane 4, LN3; lane 5, LN4. Note sharp increase in BCL-2 expression in LN3 and LN4 cells that correlates with resistance to growth inhibition in CSS (Figure 1).

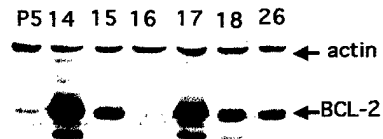


Fig. 3: BCL-2 expression in representative transfectants. P5, Pro5 parental cells. Numbers above other lanes indicate particular BCL-2 subclones. Protein loading was standardized by immunoblotting with an anti-actin antibody (arrow).

Androgen independence is associated with increased expression of BCL-2. Our preliminary experiments indicated that the LNCaP-LN3 subclone was resistant to thapsigargin- and doxorubicin-induced apoptosis compared to the LNCaP-Pro5 or the LNCaP parental cells(2). Analysis of BCL-2 polypeptide expression revealed substantially higher levels of the protein in the LNCaP-LN3 and -LN4 cells compared to the other cell lines in the panel (Figure 2). We subsequently generated transfectants of the LNCaP-Pro5 subclone expressing either empty vector (neo control) or human BCL-2 under the control of the CMV promoter (Figure 3) that we used in subsequent experiments. Preliminary experiments confirmed that the BCL-2 transfectants were resistant to thapsigargin-induced apoptosis (not shown). Overexpression of BCL-2 was associated with increased androgen-independent growth (Figure 4) and increased expression of PSA (Figure 5). Conversely, treatment of the LNCaP-LN3 cells with an antisense BCL-2-specific oligonucleotide restored androgen sensitivity to the cells (Figure 6) and inhibited constitutive PSA production (data not shown).

Constitutive AR activity increases with metastatic potential. We speculated that the androgen independence observed in the LN3 and LN4 cells would be associated with downregulation of AR expression or function. Immunoblotting experiments did not reveal consistent differences in AR expression in the LN3 and LN4 cells (Figure 7). Strikingly, however, analysis of constitutive AR activity with an ARE-driven luciferase reporter construct revealed substantially higher levels in the androgen-independent subclones (Figure 7). Similar results were obtained when a natural AR-sensitive promoter (the probasin promoter) was analyzed (data not shown).

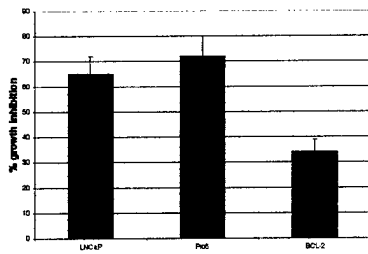


Fig. 4: BCL-2 promotes growth in charcoal-stripped serum. Growth was measured by ³H-thymidine incorporation. Results from one representative BCL-2 transfectant.

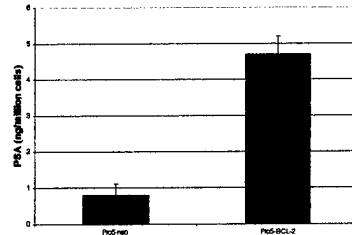


Fig. 5: BCL-2 promotes PSA production. Parental Pro5 cells or a BCL-2 transfectant were grown in normal medium for 24 h, and PSA was measured by ELISA.

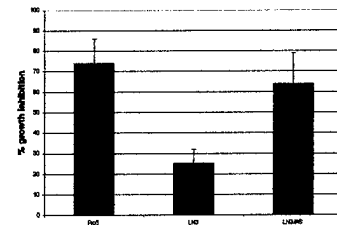


Fig. 6: An antisense BCL-2 oligonucleotide restores androgen sensitivity in LNCaP cells. Cells were pretreated with oligo for 48 h.

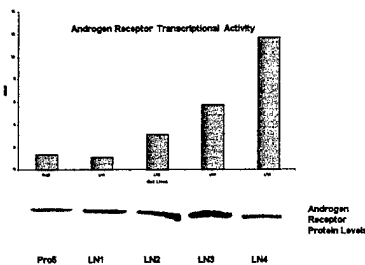


Fig. 7: AR activity (synthetic 3x ARE) and AR expression in LNCaP variants. Order: Pro5, LN1, LN2, LN3, LN4. AR activity was measured using the synthetic (ARE-dependent) and probasin luciferase promoter constructs. Strikingly, all of the BCL-2 transfectants expressed higher constitutive AR activity than the parental cells or the neomycin controls (Figure 8). AR activity was further increased by the synthetic androgen R1881 and was inhibited by Casodex. Very similar results were obtained when the probasin promoter-reporter construct was analyzed (Figure 9).

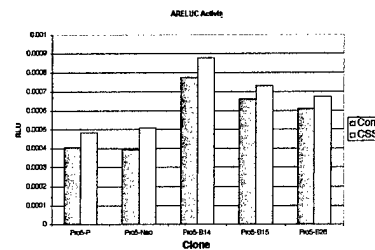


Fig. 8: AR activity (synthetic 3x ARE) in BCL-2 transfectants. Gray bars: CSS; Open bars, normal medium.

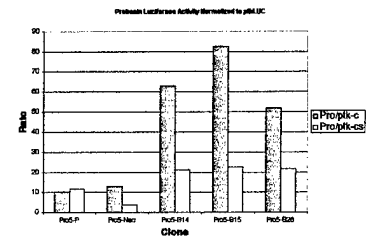
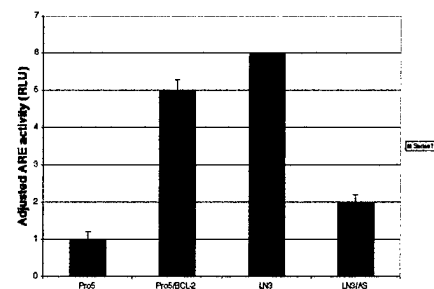


Fig. 9: Probasin promoter activity in BCL-2 transfectants. Gray bars: R1881; open bars: CSS.

AR activity is enhanced by BCL-2 expression. Prostate-specific antigen is an androgen-regulated gene (3) The observation that the LNCaP-LN3 cells and the LNCaP-Pro5 BCL-2 transfectants produced higher levels of PSA than either the parental LNCaP cells or the parental Pro5 cells suggested that AR function might be potentiated by BCL-2. To test this possibility, we measured AR activity using the synthetic (ARE-dependent) and probasin luciferase promoter constructs. Strikingly, all of the BCL-2 transfectants expressed higher constitutive AR activity than the parental cells or the neomycin controls (Figure 8). AR activity was further increased by the synthetic androgen R1881 and was inhibited by Casodex. Very similar results were obtained when the probasin promoter-reporter construct was analyzed (Figure 9).

In a final series of experiments we investigated the effects of an antisense BCL-2-specific oligonucleotide on the AR activity observed in the LNCaP-LN3 cells. Consistent with its effects on PSA production, the antisense BCL-2 oligonucleotide strongly suppressed AR-dependent promoter activity in the cells (Figure 10).

Fig. 10: Effects of AS-BCL-2 on AR activity in LN3 cells (3x synthetic ARE). Controls: Pro5 parental, Pro5-BCL-2.2. Cells were preincubated with oligo for 48 h prior to analysis.



Progress/Results of Studies in Specific Aim 2:

Here we designed experiments to test the effects of a commercially available proteasome inhibitor (MG-132) on parental LNCaP-Pro5 tumors (Task 5) and tumors derived from BCL-2 transfectants (Task 6). As pointed out in our 18 month progress report, our institution initiated a Phase I clinical trial with a new proteasome inhibitor (PS-341) that began enrolling patients soon after the start of our funding period. We therefore substituted PS-341 for MG-132 in all of our subsequent studies.

Effects of PS-341 in orthotopic human prostate cancer xenografts. In close collaboration with ProScript, we conducted eight separate toxicity and efficacy studies in nude mice bearing orthotopic human PC-3M and LNCaP-LN3 prostate tumors. Drug was delivered via intravenous (tail vein) administration in saline on a once-weekly or twice-weekly schedule. Treatment was initiated on day 7 post tumor implantation and continued until day 28, when animals were sacrificed and tumors were analyzed. The animals tolerated doses of PS-341 up to 0.8 mg/kg without toxicity. Animals dosed with 1 mg/kg displayed wasting and diarrhea, and doses above 1 mg/kg led to lethality.

Overt tumor regression was only observed at the 1 mg/kg dose level (**Figure 11**). At this dose, PS-341 was equally effective against tumors derived from parental or BCL-2-transfected cells (**Figure 11**). Analysis of PS-341-induced apoptosis by TUNEL staining (48 h post-treatment) demonstrated that the drug caused dose-dependent increases in cell death (**Figure 12**). Although tumor volumes were similar to control groups in animals treated with 0.6 mg/kg PS-341, histological analysis demonstrated extensive central necrosis in all (n = 21) drug-treated tumors (data not shown).

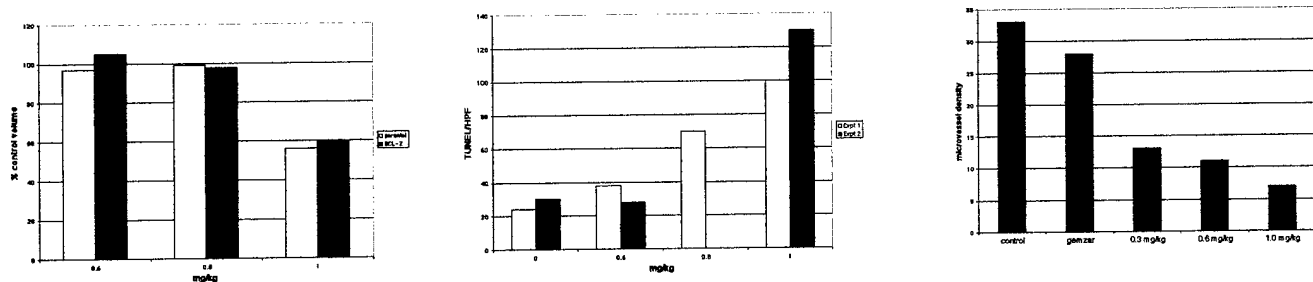


Figure 11 (left): The MTD dose of PS-341 promotes partial regression of established tumors. **Figure 12 (center):** Dose-dependent effects of PS-341 on tumor cell apoptosis in vivo. **Figure 13 (right):** Dose-dependent effects of PS-341 on tumor microvessel density. Note strong effects observed at lowest dose of drug.

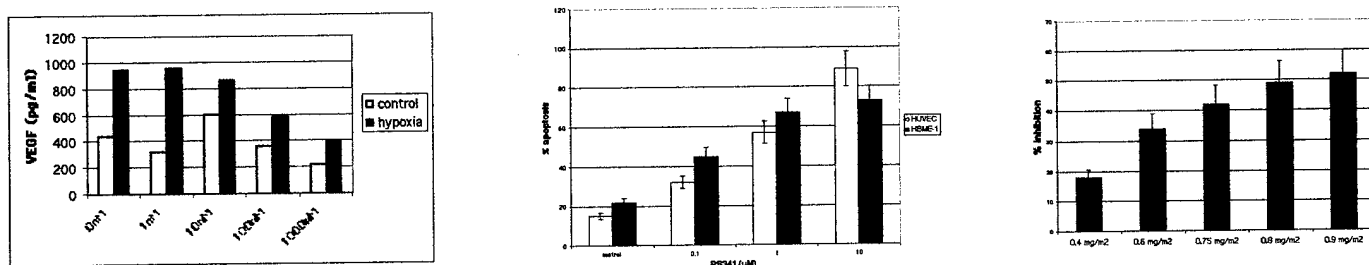


Figure 14 (left): Effects of PS341 on hypoxia-induced VEGF secretion (LNCaP-Pro5 cells). Hypoxia exposure: 24 h. **Figure 15 (center):** PS-341-induced apoptosis in endothelial cells. Cells were continuously exposed to drug for 24 h. DNA fragmentation was measured by PI/FACS. **Figure 16:** Dose-dependent inhibition of 20S proteasome activity in a Phase I trial of PS-341 in prostate cancer patients. Nearly indistinguishable results are observed in a parallel trial conducted at Memorial Sloan-Kettering Cancer Center.

Central necrosis occurs in tumors that outgrow their blood supply. We therefore wondered whether PS-341 had some indirect effect on tumor vascularity that might explain the patterns of cell death observed. Quantification of tumor microvessel density (MVD) by anti-CD31 immunohistochemistry confirmed that PS-341 treatment revealed dose-dependent inhibition (Figure 13). We are currently analyzing levels of angiogenic factors (VEGF, bFGF, and IL-8) in these tumors by immunohistochemistry. Preliminary in vitro studies confirmed that PS-341 caused a concentration-dependent inhibition of VEGF production in LNCaP-Pro5 cells exposed to hypoxia (Figure 14). Direct effects on tumor endothelial cells may also be involved, because PS-341 also induced rapid apoptosis in human HUVEC or HBME-1 vascular endothelial cells in vitro (Figure 15).

Phase I trial of PS-341 in patients with advanced (prostate) cancer. Prompted in part by our preclinical observations, Dr. Christos Papatreou at our institution implemented the first clinical trial of PS-341 in patients with androgen-independent, metastatic prostate cancer. A conservative dose escalation design was initially adopted that was later modified due to lack of toxicity. In our trial patients are treated once weekly with the drug, but a biweekly schedule is currently being evaluated in parallel trials at MSKCC and NYU. A novel assay measuring 20S proteasome activity in peripheral blood cells was employed to track biological efficacy in vivo. The result of these analyses confirmed dose-dependent inhibition of proteasome activity by the drug (Figure 16). Drug activity is reduced by 50% at 24 h and not detectable at 48 h post-treatment. We are currently accumulating patients (n = 20) at the 80% inhibition of 20S activity dose level. Although few patients have been evaluated at levels of PS-341 predicted to be efficacious (> 80% 20S inhibition), indications of clinical response have been observed. Several patients demonstrated stabilization or reduction in PSA levels ("PSA responses"), and in one patient we observed an objective (CT-based) response in a lymph node.

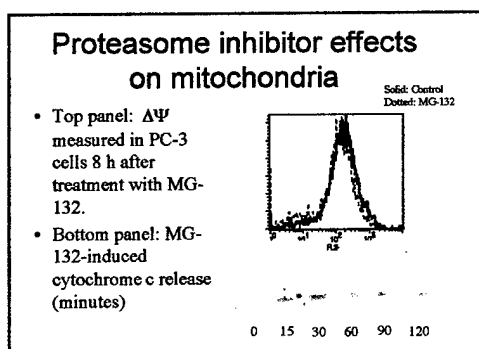


Figure 17: Effects of the proteasome inhibitor MG-132 on mitochondrial events.

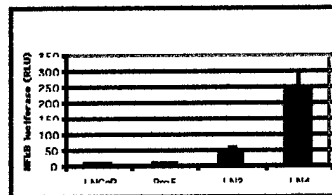


Figure 18: Constitutive NF κ B activity in LNCaP variants (10% serum, synthetic NF κ B promoter)

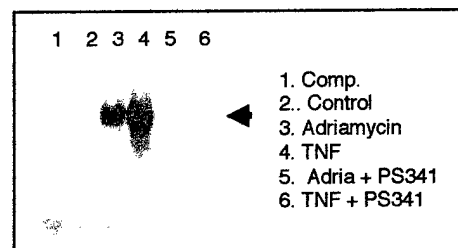


Figure 19: PS-341 blocks doxorubicin-induced NF κ B activation in PC-3 cells. NF κ B DNA binding activity was measured by EMSA.

Biochemical effects of PS-341 in prostate cancer cells. Certain features of its toxicity profile in animals suggested to us that PS-341 might have direct effects on mitochondria. To test this idea, we measured cytochrome c release and mitochondrial membrane potential in PC-3 cells exposed to PS-341 in vitro. Strikingly, the compound mobilized cytochrome c within 15 minutes (Figure 17). These kinetics are much more rapid than those observed with a large panel of other potent pro-apoptotic stimuli, including staurosporine, thapsigargin, anti-Fas, or doxorubicin. PS-341-induced cytochrome c release was slightly delayed but not abrogated in PC-3 cells transfected with BCL-2, whereas BCL-2 completely suppressed thapsigargin-induced release. Thus, we suspect that the direct cytotoxic effects of PS-341 are due to mobilization of cytochrome c.

Interestingly, proteasome inhibitors do not cause a decrease in mitochondrial membrane potential (Figure 17), suggesting that cytochrome c release is not due to a global collapse of membrane structure or function.

PS-341 blocks NFkB. Certain cancer chemotherapeutic agents have been reported to activate NFkB, leading to attenuation of drug-induced apoptosis (4). A mutant, proteasome-insensitive form of NFkB's physiological inhibitor, IkBaM, blocks this NFkB activation and dramatically sensitizes tumor cells to chemotherapy (4). Our preliminary data demonstrate that NFkB activity is elevated in the androgen-independent LNCaP variants, LN3 and LN4 (Figure 18). In addition, we have confirmed that doxorubicin activates NFkB in PC-3 cells, and that PS-341 completely blocks this response (Figure 19).

PS-341 stabilizes p53. Most conventional cancer chemotherapeutic agents are thought to induce apoptosis through a p53-dependent mechanism (5). Indeed, the effects of DNA damaging agents on cells within the NCI's 60 cell line panel correlate with wild-type p53 status (6). We have conducted single-strand conformation polymorphism (SSCP) analysis of exons 5-9 of the p53 gene in the LNCaP subclones and have confirmed that they all express wild-type p53. Furthermore, treatment with PS-341 in vitro results in concentration-dependent increases in p53 expression, as well as increases in the p53 targets, p21 and MDM-2 (Figure 20). The concentration of PS-341 required to stimulate detectable increases in p53 levels (<100 nM) is lower than the concentration required for induction of apoptosis (>1 μ M). Because p21 and MDM-2 are proteasome substrates, we do not know at present whether the increases in expression observed in PS-341-treated cells are due to transcriptional activation, protein stabilization, or both. Importantly, the PC-3 cells lack wild-type p53, yet they are nearly as sensitive to proteasome inhibitor-induced apoptosis as the LNCaP cells are. This supports our hypothesis that proteasome inhibitors do not directly kill cells via p53-mediated transcriptional activation but rather by rapid disruption of mitochondrial function.

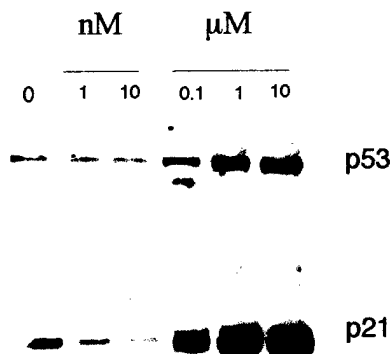


Figure 20: Dose-dependent effects of PS-341 on p53 and p21 expression in LNCaP-Pro5 cells. Incubation time: 3 h.

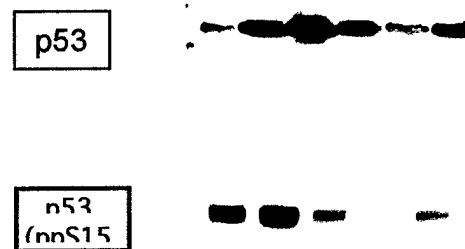


Figure 21: Effects of conventional chemotherapeutics on p53. LNCaP-Pro5 cells, exposure time = 3 h. Lanes (in sequence): Control, doxorubicin, VP16, gemcitabine, estramustine and bleomycin

Potential of drug-induced apoptosis by PS-341 in vitro. We selected five agents for our preliminary screening because of their known effects on p53 (etoposide, doxorubicin) or their relevance to current prostate cancer therapy (gemcitabine, estramustine, and taxol). To determine their effects on p53, we treated LNCaP-Pro5 cells with concentrations of each agent that induced optimal levels of apoptosis and measured p53 levels by immunoblotting. Etoposide, doxorubicin, and gemcitabine all induced substantial increases in p53

expression, and all three agents induced phosphorylation of p53 on serine 15, considered a marker for activation of the protein's transcriptional activity (**Figure 21**). In contrast, neither estramustine nor taxol promoted significant p53 stabilization or phosphorylation (**Figure 21** and data not shown).

We next evaluated the effects of a sub-lethal concentration of PS-341 (50 nM) on p53 phosphorylation and apoptosis induced by these agents. Unexpectedly, PS-341 actually inhibited serine 15 phosphorylation of p53 in cells treated with etoposide, doxorubicin, or gemcitabine (**Figure 22**). Despite this, PS-341 enhanced levels of apoptosis induced by all three agents (**Figure 23**). In contrast, the proteasome inhibitor had no effect on estramustine-induced apoptosis, and it actually inhibited apoptosis induced by taxol (**Figure 23**). These effects on the taxol response were associated with abrogation of taxol's ability to promote cell cycle arrest at the G₂/M phase transition, even though PS-341 induced arrest at this phase when applied to cells by itself (**Figure 24**).

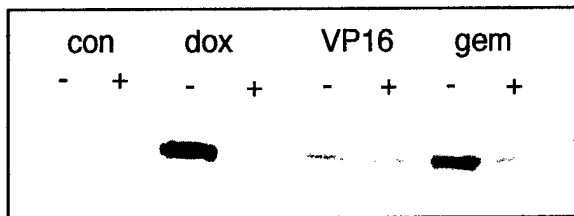


Figure 22: PS341 inhibits chemo-induced p53 phosphorylation. Pro5 cells, 3 h incubation.

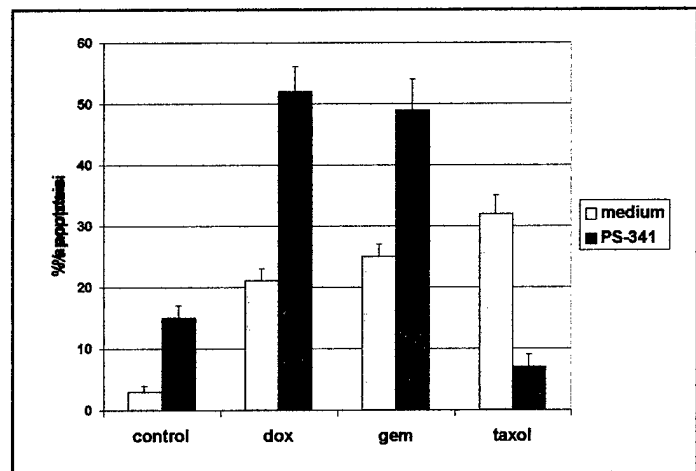


Figure 23: Effects of PS-341 on chemotherapy-induced apoptosis in vitro. LNCaP-Pro5 cells, 24 h incubation; apoptosis was measured by Hoechst staining. ADR=doxorubicin, TX=taxol, esta=estramustine.

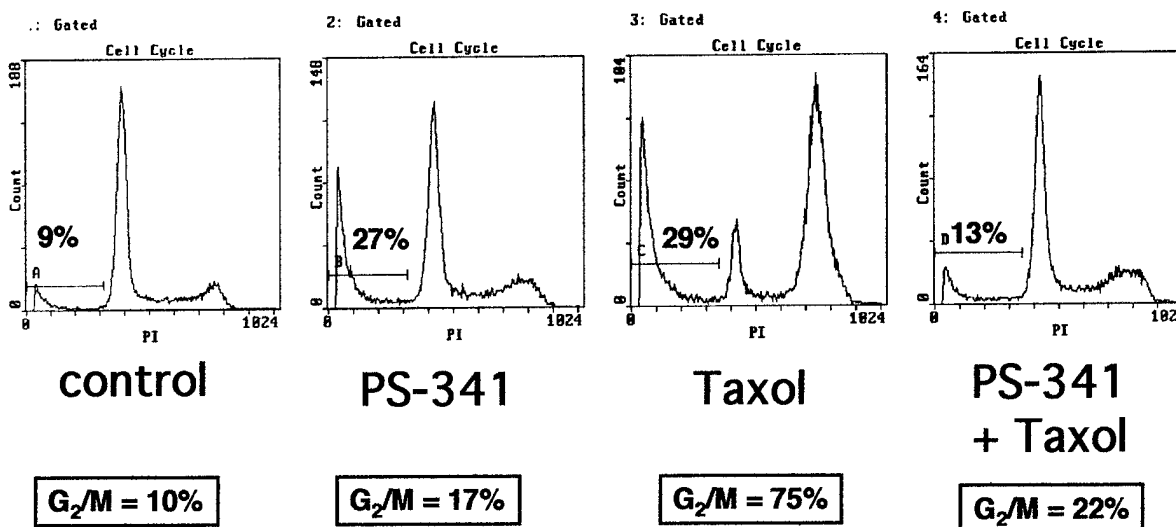


Figure 24: PS341 inhibits taxol-induced cell cycle arrest and apoptosis in LNCaP-Pro5 cells. Cells were incubated with PS-341 and/or paclitaxel for 24 h, and DNA content was measured in 0.1% Triton-permeabilized cells by propidium iodide staining and FACS analysis. Similar results were obtained in DU-145 cells. Note that in PC-3, PS-341 still interferes with cell cycle arrest but does not block apoptosis.

Evaluation of PS-341-based combination chemotherapy in xenografts. The results of our in vitro studies indicated that PS-341 increased apoptosis induced by DNA damaging agents but interfered with taxol-induced apoptosis in 2/3 cell lines tested. However, our analysis of PS-341's activity as a single agent demonstrated that it also inhibited angiogenesis, an effect that is probably distinct from tumor cell apoptosis. To test the relevance of our in vitro studies we have begun to assess the effects of PS-341-based combinations in our xenograft models. Animals bearing established (14-day) subcutaneous LNCaP-Pro5 tumors were given biweekly i.v. PS-341 (1 mg/kg) alone or in combination with biweekly i.v. gemcitabine (50 mg/kg). Alternatively, animals were dosed with biweekly PS-341 in the presence or absence of paclitaxel or docetaxel (10 mg/kg). Tumors were measured at the time of each injection and at the conclusion of the experiment. Combination therapy with gemcitabine plus PS-341 led to additive reductions in tumor volume (Figure 25), whereas drug-drug interference was observed in animals treated with the PS-341/taxoid combinations (Figure 26). These results are entirely consistent with the in vitro results. In ongoing in vitro studies we have found that the interference between PS-341 and taxoids is highly schedule-dependent and is not observed when the taxoid is delivered first (data not shown). We plan to investigate the effects of scheduling on the xenografts in future studies.

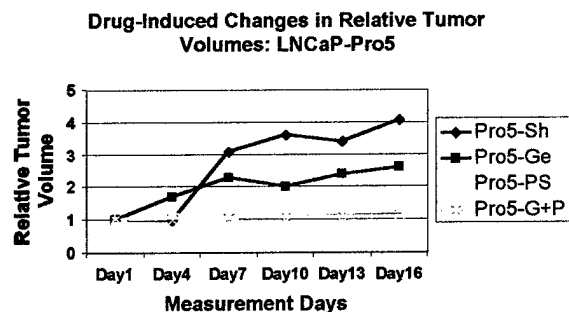


Figure 25: Effects of PS-341 on gemcitabine-induced tumor regression. See text for experimental details.

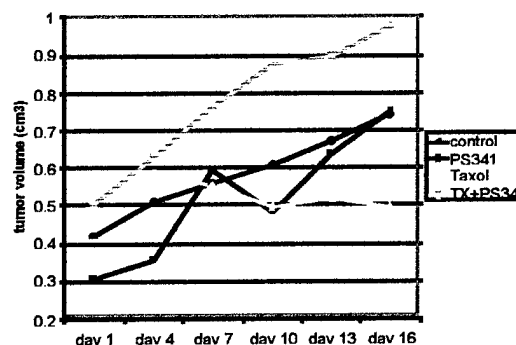


Figure 26: Effects of PS-341 on taxol-induced tumor regression. See text for experimental details.

C. Key Research Accomplishments

- Identified a novel molecular interaction between BCL-2 and the androgen receptor that may contribute to androgen-insensitive growth
- Showed that the activity of the transcription factor, NFkB, correlates with androgen independence and metastatic potential.
- Defined the effects of a new and potent chemotherapeutic agent (PS-341) on tumor cell apoptosis and angiogenesis in vivo
- Provided the first evidence that the effects of PS-341 on the activities of conventional chemotherapeutic agents will be drug- and schedule-dependent
- Stimulated the first Phase I clinical trial of PS-341 and guided NCI/CTEP and Millennium, Inc. in designing trials of PS-341 in combination with taxotere

D. Reportable Outcomes

Manuscript:

"BCL-2-mediated augmentation of androgen receptor function contributes to androgen-independent growth in LNCaP-derived human prostate cancer cells." (Attached in Appendix).

Manuscripts documenting effects of PS-341 on prostate cancer cells in vitro (#1) and in vivo (#2) are in preparation pending the outcome of a final series of experiments.

Abstracts:

C. Bruns, M.T. Harbison, R.J. Bold, P. Elliott[#], J. Adams[#], J. Abbruzzese, and C. Pettaway, and D.J. McConkey, PS-341: A NEW AGENT WITH ACTIVITY IN PROSTATE AND PANCREATIC CANCER. NCI/AACR/EORTC Joint Meeting on Cancer Chemotherapy, Washington, DC, November, 1999.

McConkey, D.J., Williams, S.A., and Papandreou, C. "Role of p53 in proteasome inhibitor-based combination chemotherapy." Annual meeting, American Association for Cancer Research, San Francisco, March 2000.

McConkey, D.J., Williams, S.A., Logothetis, C., and Papandreou, C.N. "Preclinical evaluation of proteasome inhibitor-based combination chemotherapy for advanced prostate cancer." Annual meeting, American Association for Cancer Research, New Orleans, March 2001.

Invited lectures:

"The proteasome as a new target in metastatic prostate cancer." CaP CURE Annual Retreat, September, 1998.

"PS-341: Mechanisms of action." CTEP Spring 2001 Phase I Meeting, Bethesda, MD, March 14, 2001.

Funding applied for:

"Proteasome inhibitor-based combination chemotherapy for androgen-independent prostate cancer." Principal Investigator: McConkey. DOD PCRP FY00 CDMRP.

"Phase I clinical trial of proteasome inhibitor PS-341." Principal Investigator: Papandreou. R-21, NIH/NCI, submitted 4/9/01.

Trainee support:

Simon A. Williams (predoctoral)

Victor Bondar (postdoctoral)

The award was also critical for the Principal Investigator's receiving promotion to Associate Professor with Tenure (September, 1999).

E. Conclusions

Our work on the BCL-2-androgen receptor interaction in AI-PC cells has several implications for our understanding of the biology of prostate cancer progression. Previously, most investigators had assumed that the acquisition of the androgen-independent phenotype is associated with loss of receptor function, and that some other pathway (i.e. overexpression of BCL-2) might compensate for this loss to allow the cells to survive

and divide in the absence of hormone. In this model the androgen receptor is not functionally relevant in AI-PC. Our results suggest that an alternative mechanism may be involved. Instead of substituting for the androgen receptor to promote cell proliferation and survival, BCL-2 actually promotes androgen receptor function when levels of androgen are limiting. The implication of this finding is that therapies designed to target both BCL-2 and the androgen receptor simultaneously could display significant anti-tumor activity. In addition, it is possible that BCL-2 alters the profile of androgen receptor-mediated gene expression. We plan to explore both of these possibilities in future research efforts. In addition, we will design experiments aimed at uncovering the specific molecular mechanism(s) underlying the effects of BCL-2 on androgen receptor function.

Our investigation into the efficacy and mechanisms of action of PS-341 in AI-PC has already had impact on the initiation and design of clinical trials. The observation that proteasome inhibitors bypass BCL-2-mediated cell death resistance was what generated enthusiasm for exploring its activity in patients with AI-PC. The implication of NFkB as one of their downstream targets has stimulated interest in using the drug to inhibit NFkB-regulated genes (i.e. IL-6, MMP-9, IL-8) associated with morbidity in advanced prostate cancer. Even more important were our studies with PS-341 and taxoids, which prompted CTEP to redesign its planned clinical trials with the combination to avoid drug interference. In future efforts we plan to work closely with Drs. Papandreou and Logothetis to determine whether or not the effects we have documented in our preclinical studies actually occur in patients by analyzing serial biopsies obtained before and after therapy. We submitted an R-21 proposal on these to NCI (4/9/01). We also hope to be able to investigate the molecular mechanism(s) involved in the PS-341/taxol interaction as part of a project that will be submitted as an RO1 later this year (6/1/01).

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BCL-2-mediated augmentation of androgen receptor function contributes to androgen-independent growth in LNCaP-derived human prostate cancer cells.

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___ Text pages; ___ Figures; ___ Tables.

Running Title:

Key Words:

¹ This work was supported in part by

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³ The abbreviations used are: BCL-2, B cell leukemia/lymphoma gene 2; LNCaP, lymph node cancer of the prostate; PSA, prostate-specific antigen;

ABSTRACT

Defining mechanisms of androgen-independent prostate cancer progression remains vital to improving therapeutic outcome. We evaluated the mechanism of relative androgen independent proliferation and PSA expression for in vivo-selected LNCaP-LN3 [LN3-metastatic, high Bcl-2 expression) cells compared with LNCaP-PR05 (PR05-poorly metastatic, low Bcl-2 expression)] and LNCaP parental cell lines. Measurements of in vitro growth in the presence and absence of androgen (with or without the steroid receptor antagonist bicalutamide) demonstrated that both LNCaP and PR05 cells were significantly more sensitive than LNCaP-LN3 cells to the growth inhibitory effects of androgen withdrawal. Analysis of androgen receptor (AR) mRNA and protein levels by northern and western blotting revealed that LNCaP-LN3 cells expressed lower levels of the receptor. Despite these findings, PSA mRNA expression in LNCaP-LN3 cells was consistently higher and maintained in the presence of androgen ablation in vitro when compared with parental or PR05 cells. Downregulation of Bcl-2 expression in LNCaP-LN3 cells using antisense oligonucleotides led to a reduction in PSA expression and percentage of cells in S phase in cells deprived of androgen, whereas enforced overexpression of BCL-2 in LNCaP-Pro5 cells promoted androgen-independent PSA expression and S phase cell cycle progression. Transient transfection experiments utilizing both a synthetic ([ARE]2-E1b-luciferase) promoter and the endogenous probasin gene (rPB-Luciferase) promoter were employed in LNCaP cells expressing low (PR05 line, Neo clone) and high Bcl-2 levels(LN3 line, B3, B14, and B15 clones) to directly determine if Bcl-2-mediated androgen-independent growth and PSA expression involved the androgen signaling pathway. The transcriptional activity of cells expressing high Bcl-2 levels was 2-4 fold higher in the presence, and 6-9 fold higher in the absence of androgen in vitro, compared with low Bcl-2 expressing

cells. Taken together, these data strongly suggest that androgen-independent growth of LNCaP cells overexpressing Bcl-2 is related to maintenance of proliferation that is mediated in part via the androgen signaling pathway.

INTRODUCTION

Prostate Cancer is the second leading cause of cancer related death (1). The final common pathway for patients dying of the disease is the development of distant metastases (with or without local progression) that are refractory to androgen ablation and subsequently to cytotoxic chemotherapy (2-7). Of note, many androgen independent cells continue to express a functional androgen receptor at varying levels (8-10) and are characterized by rapid growth and expression of genes that are normally regulated (or at least in part) by androgens (i.e., prostate specific antigen, human glandular kallikrein, cyclin dependent kinases, vascular endothelial growth factor etc.) (9-13).

A growing body of evidence suggests that androgen independence may arise via "adaptation" of the existing androgen receptor-signaling pathway (reviewed in reference 14). The androgen signaling pathway broadly consists of 1) the major ligands testosterone and dihydrotestosterone, 2) the androgen receptor (AR), 3) androgen responsive elements within the promoter regions of genes regulated by the pathway, and 4) transcription regulating proteins (i.e., coactivator or corepressor molecules) that either enhance or repress transcription of androgen regulated genes (14). With respect to adaptations of the normal pathway, Culig et al. (15-16) and Hobisch et al. (17) have shown that growth factors and cytokines such as Epidermal Growth Factor (EGF), Keratinocyte Growth Factor(KGF), Insulin Like Growth Factor- 1(IGF-1), Luteinizing hormone releasing hormone agonists, and Interleukin-6 are sufficient to cause the transcription of genes in the virtual absence of androgen or are synergistic with low levels of androgen. The agents listed above apparently require the presence of a functional androgen receptor as their transcriptional activity is significantly inhibited in the presence of androgen

receptor blockade (with bicalutamide) (15-17). Downstream of such growth factor -receptor interactions, crosstalk between steroid hormone signaling pathways, receptor tyrosine kinases and protein kinase signaling pathways is also involved, as tyrosine kinase inhibition with genistein, or pharmacological manipulation of the Protein kinase A, C, and mitogen activated kinase pathways affects transcriptional activity of synthetic and endogenous androgen responsive promoter reporter constructs as well as endogenous prostate specific antigen expression (18-23). The AR is a phosphoprotein with known phosphorylation sites (24-25), but , it remains to be determined whether induction of different kinase pathways directly affects AR phosphorylation or if phosphorylation of other proteins including coactivators or basal transcription factors are important in the absence of androgen (14,18-24).

Additional mechanisms of pathway adaptation include: 1) AR mutations which alter the specificity of the receptor for other ligands (i.e. estrogen, progesterone, nonsteroidal antiandrogens, adrenal androgens) (26-28), 2) AR amplification, which presumably allows maintenance of transcription in the presence of low serum androgen concentrations (29-30), and 3) alterations in AR coactivator or corepressor function number or function which alter the efficiency of gene transcription (10, 14, 31-35).

Androgen independent growth of prostate cells is also related to the cells ability to develop alternate growth and survival pathways that "bypass" the androgen-receptor signaling pathway (14). Examples include autocrine and paracrine growth factor production or overexpression of growth factor receptors such as EGF, transforming growth factor α , fibroblast growth factors (FGF), and IGF's (36-42). In PC-3 and DU-145 human prostate cancer cells

expressing autocrine growth factor loops, AR function is presumably unnecessary and the protein is not appreciably expressed (43). In addition, abnormalities of p53 tumor suppressor gene structure or function, and elevated Bcl-2 oncoprotein expression levels are strongly implicated in prostate cancer cell survival following androgen withdrawal (44-50).

Regarding Bcl-2 and androgen independent growth, we reported isolating metastatic clones of LNCaP human prostate carcinoma cells (LNCaP-LN3) subsequent to orthotopic implantation in nude mice that overexpressed Bcl-2 and exhibited relative androgen insensitivity both in vitro and (to a lesser degree) in vivo (51, 52). The phenotype of LNCaP-LN3 cells with respect to androgen dependence was similar to reports of other LNCaP cells engineered to over-express Bcl-2 (via transfection) when compared with LNCaP cells expressing lower Bcl-2 levels (49, 50, 52). In the studies reported by Beeham et al. and Raffo et al. maintenance of proliferation appeared independent of the known anti-apoptotic events ascribed to Bcl-2 (49, 50). Further, in subsequent studies we found that LNCaP-LN3 cells (spontaneous overexpression of Bcl-2) also exhibited abundant expression of two androgen regulated genes (PSA, and vascular endothelial growth factor) (51, 53). Based upon these observations, we hypothesized that maintenance of growth and expression of other androgen-regulated genes in the absence of androgen was causally related to Bcl-2 expression. In the present study we show that maintenance of cell growth under androgen depleted conditions in vitro is related to Bcl-2-induced maintenance of proliferation rather than differences in apoptotic potential. Further, this effect appears to be mediated via a novel interaction between Bcl-2 and the androgen-signaling pathway.

MATERIALS AND METHODS

Cell Lines

***In vitro* Culture Conditions.** The LNCaP human prostate cancer cell line was obtained from American Type Culture Collection (Rockville, MD). Non-metastatic (LNCAP-Pro5) and metastatic (LNCaP-LN3) variants were isolated by intraprostatic injection of LNCaP cells and sequential selection for nonmetastatic and metastatic variants (51). LNCaP cells and selected variants (LNCAP-Pro5 and LNCaP-LN3) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), L-glutamine, pyruvate, nonessential amino acids, and vitamins (Gibco-BRL, Long Island, NY). LNCaP-Pro5 cells overexpressing Bcl-2 via transfection (LNCaP-Pro5-Bcl-2.3, LNCaP-Pro5-Bcl-2.14, and LNCaP-Pro5-Bcl-2.15) or neomycin control transfected cells (LNCaP-Pro5-Neo) were cultured and maintained in above media with G418 (Gibco-BRL, Long Island, NY) at concentration of 400 $\mu\text{g/L}$.

Generation of Bcl-2 Transfected Cells. A full-length Bcl-2 cDNA containing plasmid, pCI-Bcl-2 (a gift from Dr. H. Kobayashi, and John Reed, The Burnham Institute, La Jolla, CA), was transfected into LNCaP-Pro5 cells using TransFastTM Transfection Kit (Promega, Madison, WI) according to manufacture's instructions. Forty-eight hours after transfection, cells were subcultured and grown in 1mg/ml G418 containing media. Three positive clones, designated as LNCaP-Pro5-Bcl-2.3, LNCaP-Pro5-Bcl-2.14, and LNCaP-Pro5-Bcl-2.15 were selected for further studies. A neomycin resistance gene containing plasmid pcDNA3 was transfected into

LNCaP-Pro5 cells to generate LNCaP-Pro5-Neo clones as a control following the same protocol as above.

In vitro Growth. LNCaP cells, selected variants (LNCaP-Pro5 and LNCaP-LN3) and transfected clones (LNCaP-Pro5-Bcl-2.3, LNCaP-Pro5-Bcl-2.14, and LNCaP-Pro5-Bcl-2.15 and LNCaP-Pro5-Neo) were seeded in 24 -multi-well plates at a density of 2×10^4 cells/well, in RPMI 1640 medium supplemented with 10% FBS. After 48 hours, medium was washed with HBSS and changed to modified RPMI with either 5% FBS, 5% charcoal stripped serum (CSS), 5% CSS with 1×10^{-9} M metribolone (R1881, NEN Life Science Products, Boston, MA). In some experiments, bicalutamide (nonsteroidal androgen receptor antagonist, Zeneca Pharmaceuticals, $1 \times 10^{-5} \sim 10^{-9}$ molar concentration) was utilized to define if proliferation was affected by androgen receptor blockade. Proliferation was assessed by cell counts obtained after 5 days in culture under the above conditions.

Northern Blot Analysis. LNCaP cells and selected variants (LNCaP-Pro5 and LNCaP-LN3) were seeded in 150 mm culture dishes and grown to 60% confluence in RPMI 1640 with 10% FBS. Cells were washed with Hank's balanced salt solution (HBSS, Gibco-BRL, Long Island, NY) and changed to modified RPMI with either 5% FBS, 5% CSS, 5% CSS with 1×10^{-9} DHT or 5% CSS with 1×10^{-9} R1881. Seventy-two hours later, cellular mRNA was prepared using the FastTrack™ Kit (Invitrogen, Inc., San Diego, CA). The mRNA (2ug) was electrophoresed on a 1% denaturing formaldehyde agarose gel, electrotransferred at 0.6A to a GeneScreen membrane (DuPont, Boston, MA) and UV-cross-linked with an UV Stratalinker (Stratagene, La Jolla, CA). The cDNA probes used in this analysis were a 1.3-kb *PstI* cDNA

fragment corresponding to GAPDH (an internal control), a 0.5 kb *EcoRI* cDNA fragment corresponding to the human androgen receptor (AR), and a 0.4 kb *StyI* cDNA fragment corresponding to PSA (prostate specific antigen, provided by J. T. Hsieh, Ph.D., University of Texas Southwestern Medical Center). Each cDNA was purified by agarose gel electrophoresis, recovered by Gene-Clean (BIO 101, Inc., La Jolla, CA), and radiolabeled with Rediprime (Amersham, Arlington Heights, IL) using [α - 32 P]deoxyribonucleotide triphosphates.

Western Blot Analysis for Androgen Receptor and Bcl-2 Expression. Two $\times 10^5$ LNCaP cells and selected variants (LNCaP-Pro5, LNCaP-LN3, LNCaP-Pro5-Bcl-2.14, and LNCaP-Pro5-Bcl-2.15) were seeded in 6-well culture dishes in RPMI 1640 with 10% FBS. After 48 hours, medium was washed with HBSS and changed to modified RPMI with either 5% FBS, 5% CSS, 5% CSS with 1×10^{-9} DHT, or 5% CSS with 1×10^{-9} R1881. Seventy-two hours later, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS), scraped and collected in 80 μ l lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% triton x-100, 2 mM Na_3VO_4). The soluble protein in the lysates was separated by centrifugation at 15,000 \times g for 5 min at 4° C. Protein concentration in the lysates was determined by Bio-Rad Protein Assay (Bio-Rad laboratories, Hercules, CA). After boiling, 30- μ g total protein was loaded and separated on 7.5% SDS-PAGE gel and electrophoretically transferred to a 0.45 μ m nitrocellulose membrane for 2 hours at 300 mA. The membranes were washed in blocking buffer [5% non fat dry milk in Tris buffered saline (10 mM Tris, pH 8.0 150 mM NaCl)], incubated in the appropriate primary antibody (rabbit polyclonal anti human androgen receptor antibody NCL-Arp [NOVOCASTRA, Burlingame, CA], mouse anti-human Bcl-2 monoclonal antibody [DAKO, Carpinteria, CA], or the 6C8 hamster anti-Bcl-2 antibody [provided by

Timothy J. McDonnell, The University of Texas M.D. Anderson Cancer Center (45), mouse monoclonal anti-human actin [Sigma Inc. St Louis Mo.]) and developed by enhanced chemiluminescence (ECL; Amersham, Inc, Arlington Heights, IL) after incubation with species-appropriate horseradish peroxidase-coupled secondary antibodies (also from Amersham.).

***In vitro* Analysis of Cell Cycle.** Five x 10⁴ Cells were incubated in 6-well dishes for 96 hours in media containing either 10% FBS, FBS with 10uM Casodex[®], 10% CSS, or CSS with 10pM R1881. Cells were trypsinized and resuspended in phosphate-buffered saline containing 50ug/ml of propidium iodide, 50 µg/ml sodium citrate, and 0.1% Triton-X detergent before fluorescence activated cell sorting (FACS) analysis. Nuclear histograms of DNA ploidy were generated and the percent of nuclei in the sub G₀/G₁ region, the G₀/G₁ region, S phase region, and the G₂/M phase regions was assessed.

Modulation of Bcl-2 Protein Levels using Antisense Bcl-2 Liposomal Oligonucleotides. Antisense Bcl-2 oligonucleotides (containing 2 translation initiation sites 5'CAGCGTGCGCCATCCTTCCC3') or controls with random scramble sequence were synthesized and encapsulated in liposomes as previously published (54). The levels of Bcl-2 modulation were determined by western blot analysis of protein lysates from treated cells. LNCaP-LN3 cells were seeded in 6 well plates at a density of 200,000 cells/well in 10 % FBS RPMI. After 48 hours, cells were fed with 2ml fresh 10% FBS with Modified Eagles Media (MEM) and 4 uM antisense Bcl-2 or control liposomal oligonucleotides. After 72 hours in incubation at 37 C, media were changed to modified RPMI in 5% FBS, 5% CSS, and 5% CSS with R1881 (1 x 10⁻⁹ M) and 4 µM of antisense Bcl-2 or control oligonucleotides. Seventy-two

hours later, the medium was removed and the cells were washed twice with PBS, scraped and collected in 100 μ l lysis buffer. Thirty μ g total protein was subjected to western blot analysis (see western blot methods above) and protein expression of Bcl-2 was assessed using a mouse anti-human Bcl-2 monoclonal antibody (DAKO, Carpinteria, CA).

In vitro Growth of Antisense Bcl-2 Treated Cells. To test the functional significance of Bcl-2 downmodulation, LNCaP-LN3 cells were seeded in 24 well culture dishes at a density of 10,000 cells/well in 10 % FBS of RPMI. After 48 hours, cells were fed with 0.5 ml fresh 10% FBS of MEM and treated with 4 μ M antisense Bcl-2 oligonucleotides (or control oligonucleotides with random scramble sequence). After 72 hours incubation at 37° C, media were changed to modified RPMI in 5% FBS, 5% CSS and 5% CSS + R1881 (1×10^{-9} M) with 4 μ M antisense Bcl-2 or control oligonucleotides. Cell proliferation was assessed by direct cell counts under the above conditions after 72 hours incubation.

Transfections and Luciferase Assay. To determine the basal and stimulated transcriptional activity of the androgen receptor signaling pathway in LNCaP cells, in vivo selected variants, and Bcl-2 transfectants, cells were transiently transfected with either a synthetic construct containing androgen response elements linked to a luciferase reporter, or those contained within the naturally occurring probasin gene-linked to a luciferase reporter. We utilized the (ARE) 2-E1b-luciferase DNA construct containing two androgen response elements with a minimal TATA box promoter (of the E1b gene) and a firefly luciferase reporter gene (34). In addition, a rat probasin promoter luciferase gene reporter DNA construct was kindly provided by Drs. Norman Greenberg [Baylor University, Houston TX] and Robert Matusik [Vanderbilt

University, Memphis TN]. The Probasin-luciferase reporter plasmid (rPB-luciferase) was constructed by inserting the -426 to +28 bp rat probasin promoter from the Hind III digested pBluescript SK-rPB-SV40Tag plasmid into the Hind III linearized pGL3 basic plasmid (Promega) (55). To control for transfection efficiency, the same cells were also transfected with the constitutive cytomegalovirus (pCMV-RL) or SV40 renilla luciferase (pSV40-RL) promoter-reporter constructs. In brief, cell lines were seeded in 60 mm or 6 well culture dishes in RPMI 1640 with 10% FBS at a density of 10^6 cells/dish or 2×10^5 cells/well. The monolayers were washed after 24 hours, and transfected with 5~20 ug of (ARE) 2-E1b-luciferase or rPB-luciferase and 1 ug pSV40-RL or 0.5 ug pCMV-RL using 10% lipofectin solution (Gibco-BRL, Gaithersburg, MD) for 5-15 hours. The cells were washed with PBS and changed to modified RPMI with either 5% FBS, 5% CSS, 5% CSS with 1×10^{-9} DHT or 5% CSS with 1×10^{-9} M R1881. Seventy-two hours later, the medium was removed and the cells were washed with PBS, scraped and collected in 200-ul lysis buffer. Luciferase activity was determined according to manufacture's instructions using the Dual-Luciferase Assay kit (Promega, Madison, WI).

***In vitro* Prostate Specific Antigen (PSA) Determination.** Two $\times 10^4$ cells were seeded in 24 well dishes with 10% FBS RPMI. One hundred μ l of supernatant was collected from the media and cells were counted on day 5. PSA production in the culture supernatants was detected using the Tandem-E PSA Assay kit (Hybritech, San Diego, CA) according to manufacture's instructions and normalized for the number of cells /well. PSA concentration of supernatants was expressed as ng/ 10^6 cells/ml. The limit of sensitivity of the assay is 0.2 ng PSA per ml.

STATISTICS

Differences in growth, transcriptional activity as assessed by luciferase activity, and PSA expression were compared between various groups utilizing either the student T test or one way analysis of variance (ANOVA) (56). A p value of < 0.05 was considered statistically significant.

RESULTS

In vitro Growth of LNCaP, PRO5, and LN3 Cells in the Presence or Absence of Androgen. Shown in Figure one is relative growth (assessed by cell counts) obtained from LNCaP, PRO5, and LN3 lines at day five when cultured under various conditions. The data are expressed as the percent of growth under control conditions (cells cultured in 10% FBS). As previously shown, both the LNCaP and PRO5 lines were significantly more sensitive to the growth inhibitory effects of androgen withdrawal in vitro when compared with LN3 cells (51). Subsequent to growth in CSS, LN3 cell counts retained 82 ± 7 % of control values when compared with LNCaP (56 ± 13 %) or PRO5 cell counts (21 ± 2 %) (LN3 versus PRO5, $p < 0.0001$, LN3 vs. LNCaP, $p = 0.04$). Restoration of androgen by supplementation of the culture media with R1881 resulted in increased growth for both LNCaP and PRO5 cell lines with a marginal effect if any upon LN3 cells. Furthermore, Bicalutamide (androgen receptor antagonist) inhibited the effects of exogenous androgen supplementation in LNCaP and PRO5 cells in a concentration dependent fashion (Fig. 1), whereas the growth LN3 cells appeared to be unaffected by the androgen receptor antagonist bicalutamide at comparable concentrations of the drug.

Androgen Receptor (AR) and PSA Expression in LNCaP Cell Lines. Considering the relative androgen-independence of growth observed for LN3 cells, northern and western blots were performed to determine if amplification of AR mRNA or protein in LN3 cells might explain maintenance of growth in the presence of sparse androgen concentrations in the media. In Fig. 2a northern analysis revealed greater AR mRNA expression in LNCaP and PRO5 lines

when compared with LN3 cells irrespective of whether androgen was present in the culture media. AR protein expression correlated well with mRNA expression in all three cell lines, with LN3 cells displaying lower levels of AR protein (Fig. 2b).

Results from PSA mRNA expression analyses *in vitro* are also shown in Fig. 2a. The expression levels directly correlated with the presence or absence of androgen (R1881) in LNCaP and PRO5 culture media, but appeared independent of R1881 in the LN3 line. Thus, differences in the regulation of growth and steady state PSA expression were elicited for LN3 cells compared with LNCaP and PRO5 cells subsequent to androgen withdrawal.

Bcl-2 Expression is Correlated with the Relative Androgen Independent Phenotype of LNCaP Cells. We have previously shown that LN3 cells overexpressed the anti-apoptotic protein Bcl-2 when compared with LNCaP and PRO5 cells, and that Bcl-2 expression was correlated with a relative resistance of LN3 cells to undergo apoptosis following incubation with thapsigargin or doxorubicin (52). To assess whether Bcl-2 was directly involved in androgen independent growth of LN3 cells, we treated them with a liposomal antisense Bcl-2 oligonucleotide (or control liposomal oligonucleotide) (54) *in vitro* to modulate Bcl-2 expression. Subsequent to titration studies, we found that a 4 μ M concentration of antisense Bcl-2 oligonucleotides adequately suppressed Bcl-2 protein expression in LN3 cells by western blotting (Fig. 3a). Treatment of LN3 cells with a control scrambled sequence oligonucleotide had no effect on Bcl-2 protein expression. Next we characterized the growth of LN3 cells in culture media with and without androgen using either 4 μ M antisense Bcl-2 or control oligonucleotides (Fig. 3b). Compared to LN3 cells treated for three days with the control

oligonucleotide, antisense Bcl-2 treated cells exhibited significant growth inhibition in CSS containing media (Fig. 3b, $p = 0.001$).

Further, to determine if Bcl-2 overexpression was sufficient to maintain growth in the absence of androgen in cells that initially exhibited low Bcl-2 expression, PRO5 cells were transfected with a plasmid containing the full length Bcl-2 cDNA or control plasmid (neomycin resistance gene as a selectable marker). Western analysis of Bcl-2 protein levels confirmed enhanced expression of Bcl-2 protein by LN3 parental cells and three PRO5 clones (B3, B14, B15) when compared with the PRO5 parental line or the PRO5 neo control clone (Fig. 4a).

Comparing in vitro growth of low versus high PRO5 Bcl-2 expressing clones in the presence or absence of androgen in the media revealed that growth of the PRO5 neo clone was inhibited approximately $76 \pm 3\%$ when compared with clone B3 ($25 \pm 17\%$, $p < 0.0001$), clone B14 (59 ± 11 , $p = 0.003$) or clone B15 ($41 \pm 5\%$, $p < 0.0001$) in the absence of androgen (Fig. 4b).

FACS Analysis of Apoptotic and S phase Populations in LNCaP Cell Lines and Transfected Clones. To determine the mechanism of how cell number was maintained in cells overexpressing Bcl-2 protein under androgen deprived conditions, propidium iodide staining and FACS analysis was used to compare the cell cycle profiles of cells expressing high (LN3, B3) or relatively low Bcl-2 expression (PRO5, PRO5-neo clone) under control conditions or following growth in the absence of androgen (CSS) with or without direct androgen receptor blockade (with bicalutamide) (Figs. 5a & 5b).

These studies revealed that the percent of cells within the sub G0/G1 peak (apoptotic cells) was relatively low under all conditions and in all of the cell lines (< 1- 3.5%) (Fig. 5a).

However, as shown in Fig. 5b, cells expressing high Bcl-2 levels (LN3 and PRO5-B3) exhibited a slightly higher proportion of nuclei within the S phase of the cell cycle compared with low Bcl-2 expressing cells when grown in control media (LN3 versus Neo, PRO5, $p = 0.06, 0.07$ respectively, B3 versus Neo, PRO5, $p = 0.008, 0.047$, respectively).

These differences were exaggerated when the cells were grown in the absence of androgen (CSS) or in the presence of bicalutamide (Fig. 5b). The percent of cells in S phase ranged from 20-56% of control values for the PRO5 parental and Neo clone. In contrast, the percent of cells maintained in the S phase for the LN3, and PRO5-B3 lines was 69-78% of control (LN3 versus Neo, PRO5, [with bicalutamide] $p = 0.06 - 0.012$), (B3 versus Neo, PRO5, [with bicalutamide] $0.027 - 0.002$) (LN3 versus Neo, PRO5 [with CSS] $p = 0.016 - 0.024$), B3 versus Neo, PRO5 [with CSS], $p = 0.05 - 0.007$).

In vitro PSA Expression in LNCaP-PRO5 Clones with Varying Bcl-2 Expression.

PSA protein expression was assessed via ELISA in PRO5 neo as well as clones that overexpress Bcl-2 protein both in the presence (R1881) and absence of androgen (CSS) in the media (Fig. 7). As anticipated, culture supernatant PSA levels (corrected for cell number) were higher in the presence of R1881 as compared to growth in CSS. However, compared with the neo clone the three clones with higher Bcl-2 expression (B3, B14, and B15) exhibited significantly higher PSA levels in culture supernatants both in the presence and absence of R1881 (p value, Bcl-2

overexpressing clones compared with the Neo clone in the presence ($p = 0.01 - 0.004$) and absence ($p < 0.05 - 0.002$) of R1881 (Fig. 6).

Bcl-2 Overexpression in LNCaP Cells Enhances Transcriptional Activity of the Androgen Receptor Signaling Pathway. As an initial step in defining the mechanism(s) of BCL-2's effects on AR-related biological functions, we tested the possibility that the protein directly promoted activation of synthetic (3x ARE) and endogenous (probasin) AR-driven promoters. Results were expressed as relative luciferase activity following adjustment for transfection efficiency [measured by cotransfecting cells with plasmids containing either constitutive CMV or SV40 promoter- renalia luciferase reporter constructs (CMV-RL and SV40-RL)]. The transcriptional activity of LN3 and PRO5 cells was compared under basal (CSS) or androgen stimulated conditions (R1881). The RLA of PRO5 cells under the various conditions was set at "1" and compared with LN3 cells.

RLA was enhanced 2-3 fold for LN3 cells compared to PRO5 cells utilizing either a synthetic or the endogenous probasin promoter, irrespective of the presence or absence of androgen in the media (Fig. 7a).

Similar experiments were performed utilizing the PRO5 Bcl-2 and neo transfected cells (Fig. 7b). In these studies, the rPB-Luciferase construct was cotransfected into PRO5-Neo and Bcl-2 clones along with CMV-RL. As in the above studies, the RLA of the PRO5 Bcl-2 transfectants was assessed and compared with the PRO5 neo clone (RLA set at "1") (Fig. 7b) under basal and androgen stimulated conditions. Bcl-2 clones exhibited significantly greater

RLA in both the presence and absence of androgen. This was especially true under androgen depleted conditions where transcriptional activity was 6-9 fold greater than in the Neo control clone (Fig. 7b). Thus overexpression of Bcl-2 protein was causally related to increased transcription of genes regulated via the androgen-signaling pathway.

DISCUSSION

Unraveling the complexity of androgen independent prostate cancer (AIPC) progression remains vital to improving the therapeutic outcome for patients with advanced prostate cancer. Independence from the normal steroid hormone/receptor signaling cascade in prostate cancer can result from "adaptations" of the normal pathway in addition to compensatory mechanisms that appear "independent" of steroid hormone signaling mechanisms (14).

One such pathway of escape involves suppression of apoptosis via overexpression of Bcl-2 protein either constitutively or subsequent to castration (46 - 48, 57). Direct evidence for the involvement of Bcl-2 in androgen independent progression was provided by Raffo et al. who showed that LNCaP cells engineered to overexpress Bcl-2 via transfection were capable of growing in castrate hosts compared with control transfected cells (49). Of note, enhanced in vitro growth of the same cells in CSS was noted and ascribed to a probable independent proliferative effect of Bcl-2 (49). Beham et al. (50) also confirmed that LNCaP cells overexpressing Bcl-2 grew faster in nude mice compared with controls. Subsequent to castration, they noted both decreased rates of apoptosis *and* increased rates of proliferation (as judged by proliferating nuclear antigen expression) in vivo (50). In patient specimens, Matshushima et al. found that 38% of prostate cancers expressed Bcl-2 protein in patients that were previously untreated and this was correlated with higher proliferation indices (subsequent to FACS analysis) and poorer disease specific survival (57). Thus, both decreased rates of apoptosis *and* enhanced proliferation

are associated with Bcl-2 expression in prostate cancer and appear relevant to the process of androgen-independent prostate cancer progression.

We have previously shown that metastatic LNCaP-LN3 prostate cancer cells overexpress Bcl-2 (51-52). In the present study, LNCaP-LN3 cells were resistant to the growth inhibitory effects of androgen withdrawal in vitro compared with the LNCaP parental or prostate selected LNCaP-PRO5 cells (Fig. 1). In addition, AR blockade using the nonsteroidal antiandrogen bicalutamide had no detrimental effect upon in vitro growth of LNCaP-LN3 cells. These data suggested that in LNCaP-LN3 cells, typical steroid hormone/receptor interactions were no longer required for maintenance of proliferation.

To determine, however, if AR amplification in the presence of minute concentrations of androgen could explain the above observations, androgen receptor mRNA and protein levels were directly measured by Northern and Western blotting, respectively (Figs. 2a & 2b). This was relevant as Visakorpi et al. had previously demonstrated AR amplification in a subset of human prostate cancers obtained from patients with clinically defined AIPC progression (29-30). To the contrary, LNCaP-LN3 cells exhibited lower steady state levels of mRNA and protein. Thus, enhanced AR protein expression did not explain the relative androgen independent phenotype of LNCaP-LN3 cells.

Prostate specific antigen expression is also androgen-regulated (11). We have previously shown that LNCaP-LN3 cells exhibited higher PSA levels in culture supernatants compared to the LNCaP or LNCaP-PR05 cell lines (51). In the present study, PSA mRNA expression levels

were compared for the three cell lines both in the presence and absence of androgen and enhanced PSA mRNA expression in LNCaP-LN3 cells was found (Fig. 2a). Of interest, PSA mRNA expression remained stable in LNCaP-LN3 cells grown in CSS in comparison to LNCaP or LNCaP-PR05 cells grown under the same conditions where decreased PSA mRNA expression was evident (Fig 2a, lanes 2, 5, and 8). Thus, in the case of LNCaP -LN3 cells both PSA expression and prostate growth appeared relatively independent of androgen when compared with the parental or PR05 cell lines.

To address the potential role of Bcl-2 in the acquisition of androgen independence we downmodulated the expression of Bcl-2 protein transiently in LNCaP-LN3 cells using specific antisense oligonucleotides (Fig. 3a). Subsequent growth assays using cells exposed to antisense Bcl-2 oligonucleotides revealed that downmodulation of Bcl-2 expression was directly correlated with significant growth inhibition in androgen depleted media (Fig. 3b). We could not however demonstrate a consistent difference in PSA expression for LNCaP-LN3 cells subsequent to Bcl-2 modulation using antisense oligonucleotides (data not shown). This may have been due to the transient nature of Bcl-2 modulation and (or) the inefficiency of antisense oligonucleotide uptake.

Subsequently, we generated stable Bcl-2 over-expression in LNCaP-PR05 cells via transfection and selected several clones for further study (Fig. 4a). When compared to the control clone (LNCaP-PR05-Neo), LNCaP-PR05 cells overexpressing Bcl-2 were capable of maintaining growth to a significantly greater extent in CSS (Fig. 4b, $p = 0.0026 - 0.0001$).

Relative maintenance of cell number in the face of androgen depletion could have resulted from either decreased rates of apoptosis or maintenance of proliferation. We directly assessed both possibilities using FACS analysis of propidium iodide stained nuclei from either high (LNCaP-LN3, clone B3) or low (LNCaP-PR05, clone Neo) Bcl-2 expressing cells. No significant differences in apoptotic potential were noted for cells expressing varying levels of Bcl-2 protein under control, androgen depleted conditions, or in the presence of AR blockade with bicalutamide (Fig. 5a). However, there were significant differences in the steady-state percentages of cells within the S phase of the cell cycle that correlated Bcl-2 protein levels, especially when cells were cultured under androgen-depleted conditions or in the presence of bicalutamide (Fig. 5b). Taken together these data strongly suggest that increasing Bcl-2 expression was directly implicated in androgen independent growth of LNCaP cells, and that the mechanism was related to maintenance of proliferation as opposed to differences in apoptotic potential.

Utilizing the stable Bcl-2 or control transfected LNCaP-PR05 cells, we subsequently measured PSA protein expression in culture supernatants both in the presence and absence of androgen (Fig. 7). Irrespective of culture conditions, normalized supernatant PSA levels were modestly, but significantly higher for clones expressing high Bcl-2 levels compared with the Neo control.

As two different processes that were normally androgen regulated (growth, PSA production) appeared to be enhanced in cells expressing high Bcl-2 levels, we conducted additional experiments to determine whether Bcl-2 had direct effects on androgen-responsive

promoters. In these studies luciferase expression was driven by either a synthetic ([ARE]2-E1b-luciferase) or endogenous (probasin:rPB-Luciferase) promoter, each of which contains functional, consensus androgen response elements. The transcriptional activities of both promoters were enhanced 2-4 fold in LNCaP-LN3 cells compared to LNCaP-PR05 in both the presence and absence of androgen in the assay (Fig. 6a). Similarly, transient transfections using the rPB-Luciferase promoter-reporter construct in LNCaP-PR05 clones with high Bcl-2 expression revealed 6-9 fold greater luciferase expression in the absence of androgen and 2-6 fold greater luciferase expression in the presence of androgen compared with the Neo control (Fig. 6b).

The findings of this study reveal a novel role for Bcl-2 protein in the progression of prostate cancer. In LNCaP-derived cells, enhanced Bcl-2 expression is directly related to maintenance of proliferation and PSA expression subsequent to androgen ablation in vitro. Furthermore, enhanced Bcl-2 expression is directly related to enhanced transcriptional activity of androgen responsive promoters. Thus, we provide evidence that Bcl-2 protein "interacts" with a functional androgen signaling pathway downstream of steroid to maintain transcription of androgen regulated genes under castrate conditions. The mechanism for such an interaction is intriguing and is currently under study. Whether Bcl-2 interacts directly or indirectly with the AR (i.e., similar to EGF, IGF-1, IL-6 or protein kinases) or coactivating proteins to enhance transcriptional activity of the pathway is unknown and remains to be determined. However, recent data from Froesh et al. have determined that the Bcl-2 binding protein BAG-1L also binds to the AR and enhances its transcriptional activity in the presence of minimal concentrations of androgen (58). Furthermore, BAG-1 has also been shown to bind to and activate Raf-1, an

upstream activator of mitogen-activated protein kinase also implicated in ligand independent AR activation (21, 59). Thus, the mechanisms of Bcl-2 action may be complex and could involve crosstalk between BCL-2-binding proteins and protein kinase-based signaling pathways (21, 58, 59).

Considering that Bcl-2 is overexpressed in 33-50% of androgen-independent bone metastases, 77-100% of androgen independent soft tissue sites, and in 12-32% of androgen dependent prostate cancers, strategies to modulate Bcl-2 expression in vivo may prove valuable (46-48, 60-62). Further, as most clinically defined "androgen independent" prostate cancers exhibit significant AR expression, strategies that inhibit downstream mediators of the AR signaling cascade in addition to Bcl-2 inhibition could prove additive or synergistic in targeting AIPC (9-10).

FIGURE LEGENDS

Figure 1. Relative growth rate of LNCaP cells and selected variants over 5 days in 5% FBS, 5% CSS or 5% CSS with R1881, and with different molar concentration of bicalutamide. Proliferative responses of the cells were assessed by direct cell counting. Bic = bicalutamide. R1881 = 10^{-9} M.

Data represent the mean \pm sd. of triplicate wells. One of three experiments shown. LNCaP-LN3 maintenance of cell growth in CSS compared with control (FBS) greater than PRO5 or LNCaP ($p < 0.0001$, $p = 0.039$, respectively).

Figure 2. *Invitro Androgen Receptor (AR) And PSA Expression In LNCaP and Selected Variant Cell Lines.*

a) *Northern blot analysis of androgen receptor and PSA mRNA expression in LNCaP cells and in vivo-selected variants. Cells were cultured under conditions of 5% FBS (lines 1, 4, and 7), 5% CSS (lines 2, 5, and 8), and 5% CSS + R1881 (lines 3, 6, and 9) over 48 hours. Cellular mRNA was extracted, subjected to electrophoresis, and hybridized with a 1.3-kb PstI cDNA fragment corresponding to GAPDH, a 0.5 kb EcoRI cDNA fragment corresponding to the human AR and a 0.4 kb StyI cDNA fragment corresponding to PSA.*

b) *AR protein expression in LNCaP and in vivo selected cell lines cultured under conditions of 5% FBS (lines 1, 4 and 7), 5% CSS (lines 2, 5 and 8), and 5% CSS + R1881 (lines 3, 6, and 9). Western blots of AR expression were*

performed with a rabbit anti-human AR polyclonal antibody utilizing actin expression as a control for loading.

Figure 3. *Modulation of Bcl-2 Protein Expression Invitro.*

- a) LNCaP-LN3 cells were treated with liposomal antisense Bcl-2 or control oligonucleotides over 72 hours. Expression of Bcl-2 protein in antisense treated and control cells was analyzed by western blotting using a mouse anti-human Bcl-2 monoclonal antibody.*
- b) Relative growth of LNCaP-LN3 cells treated with antisense Bcl-2 or control oligonucleotides. Cells were treated with 4 μ M antisense Bcl-2 or control oligonucleotides in 10% FBS RPMI over 72 hours and incubated in 5% FBS, 5% CSS or 5% CSS with R1881 for another 72 hours. Proliferative responses were assessed by direct cell counting. Results represent mean \pm sd of three experiments, (* $p < 0.0001$, growth of Bcl-2 antisense treated cells significantly inhibited in CSS compared to control treated cells).*

Figure 4. *Bcl-2 Protein Expression and Growth in the Absence of Androgen in vitro.*

- a) Expression of Bcl-2 protein in LNCaP invivo selected cells and LNCaP-PRO5 transfectants. A full length Bcl-2 cDNA containing plasmid pCI-Bcl-2 or a neomycin resistance gene containing plasmid pCDNA3 was transfected into LNCaP-Pro5 cells. Four clones, designated as LNCaP-Pro5-Bcl-2.3, LNCaP-Pro5-Bcl-2.14, LNCaP-Pro5-Bcl-2.15, and LNCaP-PRO5-neo were selected for further studies. LNCaP-LN3, PRO5 parental, and the above transfected*

cells were grown in RPMI with 10% FBS, lysed, and probed for Bcl-2 expression by western blotting.

- b) Growth of LNCaP-Pro5 transfectants in the presence or absence of androgen. LNCaP-Pro5 transfectants were cultured in 5% FBS, 5% CSS or 5% CSS with R1881 and proliferation was assessed by cell counts after 5 days. R1881 = 10^{-9} M. Results represent mean of three experiments. * $p = 0.0026 - 0.0001$, growth inhibition of neo clone in CSS greater than Bcl-2 overexpressing clones.

Figure 5. FACS Analysis of LNCaP Variant Cells and Transfectants.

- a) Analysis of SubG0/G1 Population. Cells were incubated for 96 hrs in media containing 10% FBS (control), FBS with 10uM Bicalutamide, or charcoal-stripped serum (CSS). They were trypsinized and resuspended in a solution of propidium iodide and Triton-X detergent before being subjected to FACS analysis. No significant differences in apoptotic rates noted.
- b) Analysis of S-Phase population. Cells were incubated as above and prepared for FACS analysis. Results represent the mean of three experiments (a & b).
- * % of cells in S phase significantly greater than Pro5 or Neo cell line.
- + % of cells in S phase significantly greater than Neo cell line.

Figure 6. In vitro PSA production by LNCaP-Pro5 Transfectants. PSA production was assayed by using 100 ul of supernatant collected from media after 48 hours culture in 5% CSS or 5% CSS + 10^{-9} M R1881. PSA concentration was detected

using Tandem-E PSA Assay kit (limit of sensitivity 0.2 ng PSA/ml) and normalized to ng/10⁶ cells/ml. Supernatant PSA concentrations significantly greater for Bcl-2 overexpressing clones compared with the Neo clone in the presence (⁺ p = 0.01 - 0.004) and absence (* p < 0.05 - 0.002) of R1881.

Figure 7. Transcriptional Activity of LNCaP Variants and Transfected Cells.

- a) Cells were co-transfected with (ARE)₂-E1b-Luciferase or rPB-Luciferase constructs and Renilla-Luciferase with SV40 or CMV promoters (internal control) using lipofectin. The transfected cells were incubated in 5% CSS and 5% CSS + R1881 over 24 hours and subjected to the Dual-Luciferase assay (R1881 = 10⁻⁹ M). The transcriptional activity of LNCaP-Pro5 cells under various conditions was set at 1. Results represent fold activity of LNCaP-LN3 cells above that of PRO5 cells. Mean of three experiments.

- b) Transcriptional activity of LNCaP-Pro5 Bcl-2 transfectants. Cells were transfected with rPB-Luciferase and CMV promoter- Renilla-Luciferase constructs using lipofectin method. Transfected cells were incubated in 5% CSS and 5% CSS + R1881 over 24 hours and subjected to the Dual-Luciferase assay. Transcriptional activity of LNCaP-Pro5-Neo under various conditions was set at 1. Results represent mean of three experiments.

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